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TITLE: SANGUINARINE: A NOVEL AGENT AGAINST PROSTATE CANCER

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**14. ABSTRACT**
The traditional therapeutic and surgical approaches have not been successful in the management of prostate cancer (CaP). Natural plant-based products have shown promise as anticancer agents. Sanguinarine, a benzophenanthridine alkaloid derived from the root of Sanguinaria Canadensis, has been shown to possess anti-microbial, antioxidant and anti-inflammatory properties. Our earlier studies suggested that sanguinarine may be developed as an agent for the management of prostate cancer. Based on this rationale, funded by the DOD (Award - W81XWH-04-1-0220), we initiated a study to investigate the hypothesis that sanguinarine will impart antiproliferative effects against prostate cancer via a modulation in NF-kB-pathway-mediated apoptosis. During the funding period, we have made reasonable progress towards our goals. However, the progress during this reporting period was hampered due to several unforeseen circumstances. Because of this reason, a one-year extension of the grant was also obtained in January 2007. So far, the key accomplishments of our project are as follows. We have demonstrated that sanguinarine possesses anti-proliferative effects against CaP in an athymic nude mice xenograft model. Further, our data suggested that sanguinarine-caused effects may be mediated via modulations in NF-B pathway and cyclin kinase inhibitor-cyclin-cyclin dependent kinase machinery. Our study with transgenic TRAMP model has suggested that sanguinarine may be developed as an agent for the management of CaP.

**15. SUBJECT TERMS**
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**Table of Contents**

## Introduction:

In American men, **Ca**ncer of the **P**rostate (**CaP**), continues to be one of the most frequently occurring malignancies, representing ~29% of all new cancer cases (1). The traditional surgery and therapy has not been successful in the management of CaP. Therefore, the search for novel agents and approaches for the treatment of CaP continues. Natural plant-based products have shown promise as anticancer agents. Ideally, the anti-cancer drugs should specifically target the neoplastic cells with minimal "collateral damage" to normal cells. Thus, the agents, which can eliminate the cancerous cells without affecting the normal cells, may have therapeutic advantage for the elimination of cancer cells. Sanguinarine (13-methyl[1,3]benzodioxolo[5,6-c]-1,3-dioxolo[4,5-i]phenanthridinium), derived from the root of *Sanguinaria Canadensis* and other poppy-fumaria species, is a benzophenanthridine alkaloid and a structural homologue of chelerythrine and has been shown to possess anti-microbial antioxidant and anti-inflammatory properties (2-4). Our published and preliminary studies have suggested that sanguinarine may be developed as an agent for the management of prostate cancer (5-8). Based on this rationale, funded by the Department of Defense (DOD; Idea Development Award - W81XWH-04-1-0220), we initiated a study to investigate the hypothesis that *sanguinarine will impart antiproliferative effects against prostate cancer via a modulation in NF-κB-pathway-mediated apoptosis*. Overall, we have made significant progress towards achieving our goals.

## Main Body of the Progress Report:

A brief description of the progress made during the funding period is presented in the following pages.

### *Evaluation of the anti-proliferative effects of sanguinarine against prostate cancer cells.*

We first extended our preliminary data on which this proposal was based. This resulted in a publication in "Molecular Cancer Therapeutics' (7, **Appendix-1**). The abstract of this study is produced verbatim below.

*Sanguinarine causes cell cycle blockade and apoptosis of human prostate carcinoma cells via modulation of cyclin kinase inhibitor-cyclin-cyclin-dependent kinase machinery.Adhami VM, Aziz MH, Reagan-Shaw SR, Nihal M, Mukhtar H, Ahmad N. Department of Dermatology, University of Wisconsin Medical Science Center, 1300 University Avenue, Madison, WI 53706, USA. Mol Cancer Ther. 2004 Aug;3(8):933-40.*

Prostate cancer is the second leading cause of cancer-related deaths in males in the United States. This warrants the development of novel mechanism-based strategies for the prevention and/or treatment of prostate cancer. Several studies have shown that plant-derived alkaloids possess remarkable anticancer effects. Sanguinarine, an alkaloid derived from the bloodroot plant Sanguinaria canadensis, has been shown to possess antimicrobial, anti-inflammatory, and antioxidant properties. Previously, we have shown that sanguinarine possesses strong antiproliferative and proapoptotic properties against human epidermoid carcinoma A431 cells and immortalized human HaCaT keratinocytes. Here, employing androgen-responsive human prostate carcinoma LNCaP cells and androgen-unresponsive human prostate carcinoma DU145 cells, we studied the antiproliferative properties of sanguinarine against prostate cancer. Sanguinarine (0.1-2 micromol/L) treatment of LNCaP and DU145 cells for 24 hours resulted in dose-dependent (1) inhibition of cell growth [as evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay], (2) arrest of cells in G0-G1 phase of the cell cycle (as assessed by DNA cell cycle analysis), and (3) induction of apoptosis (as evaluated by DNA ladder formation and flow cytometry). To define the mechanism of antiproliferative effects of sanguinarine against prostate cancer, we studied the effect of sanguinarine on critical molecular events known to regulate the cell cycle and the apoptotic machinery. Immunoblot analysis showed that sanguinarine treatment of both LNCaP and DU145 cells resulted in significant (1) induction of cyclin kinase inhibitors p21/WAF1 and p27/KIP1; (2) down-regulation of cyclin E, D1, and D2; and (3) down-regulation of cyclin-dependent kinase 2, 4, and 6. A highlight of this study was the fact that sanguinarine induced growth inhibitory and antiproliferative effects in human prostate carcinoma cells irrespective of their androgen status. To our knowledge, this is the first study showing the involvement of cyclin kinase inhibitor-cyclin-cyclin-dependent kinase machinery during cell cycle arrest and apoptosis of prostate cancer cells by sanguinarine. These results suggest that sanguinarine may be developed as an agent for the management of prostate cancer.

***Evaluation of anti-proliferative effects of sanguinarine against prostate cancer development in nude mice xenografts.***

Next, we conducted studies to determine the efficacy of sanguinarine against prostate cancer development and progression in athymic nude mice implanted with human prostate cancer cells. Further, we conducted experiments to determine the molecular mechanism associated with the observed anti-proliferative effects of sanguinarine. This study was presented at the 2005 Annual Meeting of the American Association for Cancer Research (April 16-20; Anaheim CA; **Appendix-2**). A brief account of this study is given below.

***Study Design:***

To determine the chemopreventive and therapeutic potential of sanguinarine against CaP in vivo, we employed the athymic nude mice xenografts model. For this experiment, the athymic (nu/nu) male nude mice (obtained from NxGen Biosciences, San Diego, CA) were randomly divided into different groups of 10 animals each and CW22Rv1 cells ($1x10^6$ cells in 50 µl RPMI + 50 µl Matrigel) were implanted in athymic nude mice by a sub-cutaneous injection on left and right sides, below the shoulders (2 tumors/mouse). The rationale for the choice of CWR22Rv1 cells is based on the fact that our major goal was to determine the chemopreventive effects of sanguinarine in early stages of CaP development, when the disease is androgen-dependent. Another reason for employing CWR22Rv1 cells was that they make PSA, which is arguably considered a *gold standard* for monitoring the CaP in humans. The animals were treated with sanguinarine (1 or 5 mg/kg body weight in 0.2 ml PBS, five days a week) by intra-peritoneal injection either one week post cell implantation to establish the preventive potential or after the development of a sizable tumor (200 $mm^3$) to examine the therapeutic potential. Thus, two different protocols were employed.

The detail of **Protocol-1** is as follows:

**Group I: Control** – cells were implanted (at the start of the experiment; day 0), no further treatment given;
**Group II: Sanguinarine (1 mg/kg)** – cells were implanted on day 0 and the mice were injected with sanguinarine (1mg/kg body weight; *i. p.*) 5 days/ week (Monday – Friday);
**Group III: Sanguinarine (5 mg/kg)** – cells were implanted on day 0 and the mice were injected with sanguinarine (5mg/kg body weight; *i. p.*) 5 days/ week (Monday – Friday)

The detail of **Protocol-2** is as follows:

**Group 1: Control** – cells were implanted (at the start of the experiment), no further treatment was given;
**Group 2: Sanguinarine (1 mg/kg)** – cells were implanted and the tumors were allowed to grow and achieve a volume of 200 $mm^3$, when the treatment with sanguinarine (1mg/kg body weight; *i. p.*; 5 days/ week – Monday – Friday) was started and continued until the termination of experiment;
**Group 3: Sanguinarine (5 mg/kg)** – cells were implanted and the tumors were allowed to grow and achieve a volume of 200 $mm^3$, when the treatment with sanguinarine (5mg/kg body weight; *i. p.*; 5 days/ week – Monday – Friday) was started and continued until the termination of experiment;

In both the protocols, the control animals received vehicle only. The treatment schedule was continued until the tumors reached a volume of 1000 $mm^3$. At this point, the animals were withdrawn from the study and euthanized. Throughout the experiment the animals were housed under standard housing conditions and had free access to autoclaved laboratory chow diet. In this protocol, to assess the possibility of treatment-toxicity, the effect of treatments on food/water consumption and body weight was monitored twice weekly throughout the study. Further, blood was withdrawn periodically to determine the effects of treatments on PSA levels in serum. The effect of sanguinarine treatment was determined on the growth of implanted tumors and the serum levels of prostate specific antigen (PSA).

At the termination of experiment, the tumors were harvested for further studies to evaluate the mechanism of the antiproliferative effects of sanguinarine against prostate cancer.

***Results & Conclusion:***

**Anti-proliferative Effects of Sanguinarine Against CaP:** As shown in figures 1 and 2, our data demonstrated that sanguinarine (both pre- and post- treatments) resulted in a highly significant inhibition in the rate of tumor growth as assessed by a regression analysis. Further, the Kaplan-Meier Analysis demonstrated that in sanguinarine treated animals (post-treatment), the rate of tumor growth (to reach to a 1000 mm3 target volume) was significantly delayed. Furthermore, treatment of mice with sanguinarine (both pre- and post- tumor) resulted in a significant reduction in serum levels of prostate-specific antigen (PSA) in nude mice implanted with CWR22Rv1 cells.

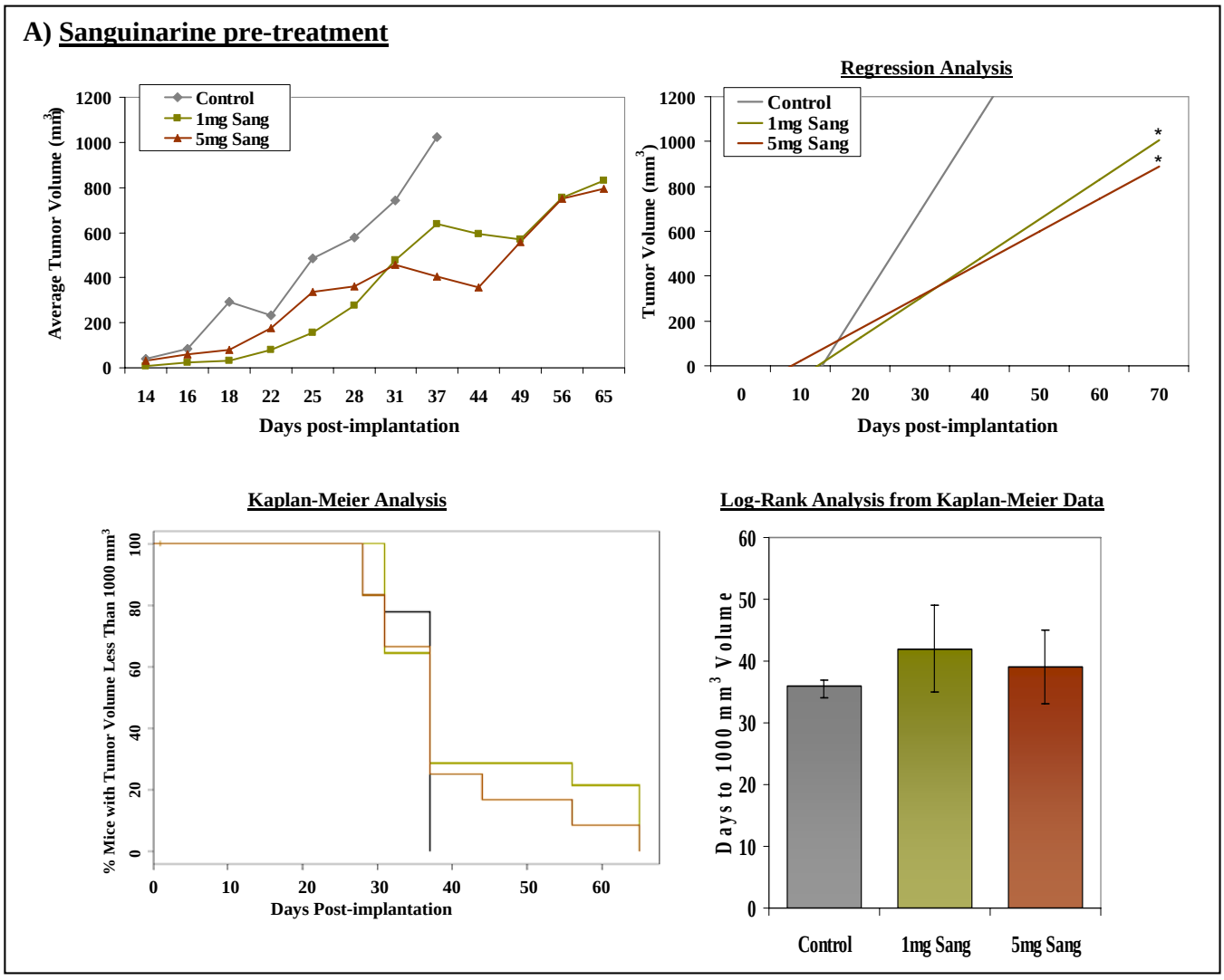


**Fig. 1: Effects of sanguinarine treatment on the growth of CWR22Rv1 cell- implanted prostate tumors in athymic nude mice.** The cells (in matrigel) were implanted on both flanks of nude mice. The animals were treated with sanguinarine as described above. The effect of sanguinarine pre-treatment was measured in terms of average tumor volume of as a function of time. Further, the rate of tumor growth was assessed by linear regression analysis. Tumor-free survival was assessed by Kaplan-Meier plot and the average time to reach 1000 $mm^3$ tumor volumes was assessed by Log-Rank analysis of Kaplan-Meier data. *$p<0.05$ was considered significant.

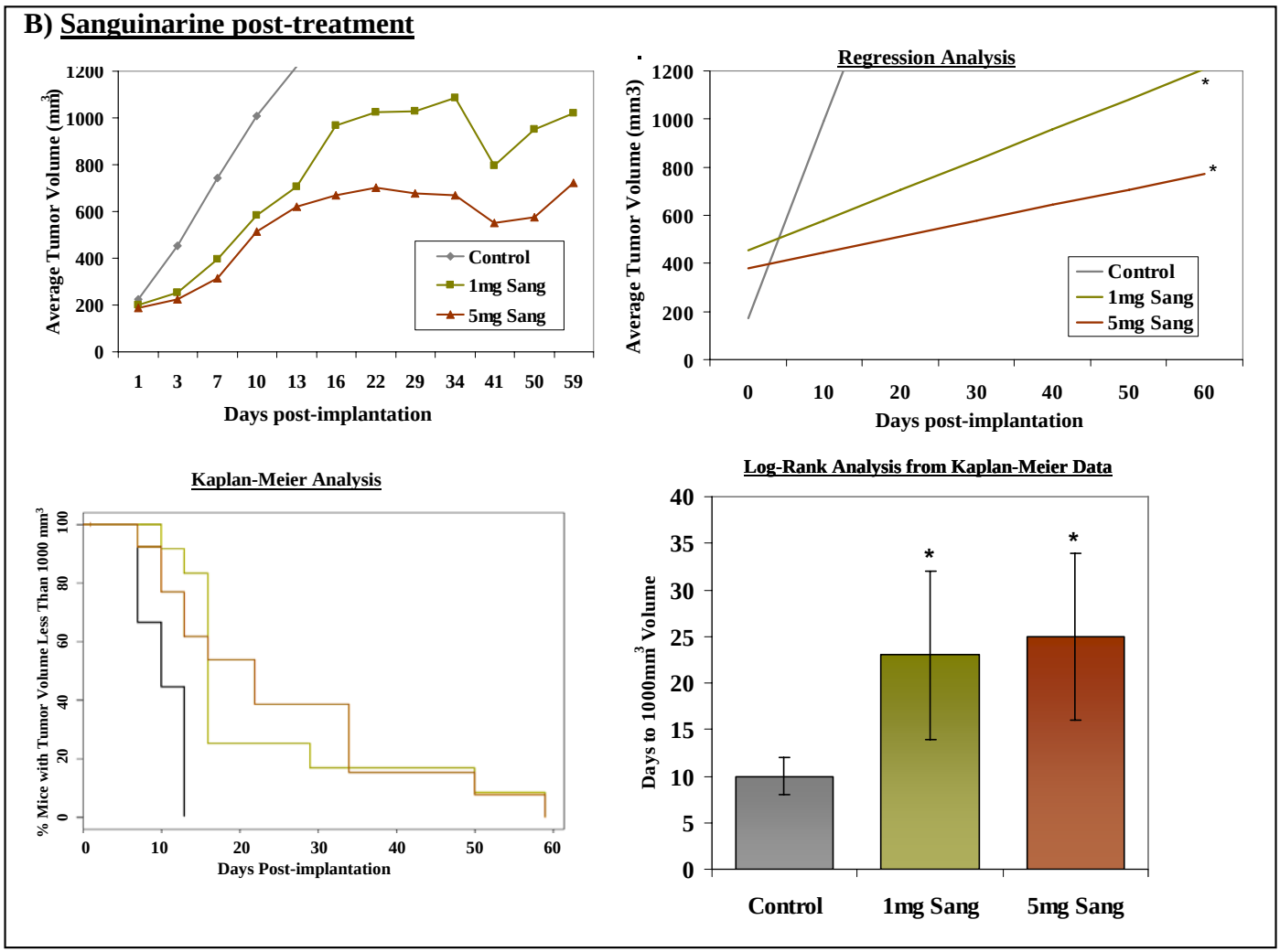


**Fig. 2: Effects of sanguinarine post-treatment on the growth of CWR22Rv1 cell- implanted prostate tumors in athymic nude mice.** The cells (in matrigel) were implanted on both flanks of nude mice. The animals were treated with sanguinarine as described above. The effect of sanguinarine treatment on the growth of established tumors was measured in terms of average tumor volume of as a function of time. Further, the rate of tumor growth was assessed by linear regression analysis. Tumor-free survival was assessed by Kaplan-Meier plot and the average time to reach 1000 $mm^3$ tumor volumes was assessed by Log-Rank analysis of Kaplan-Meier data. *$p<0.05$ was considered significant.

**Effect of Sanguinarine on Serum PSA:** Further, as described earlier, for this study, we used CWR22Rv1 cell because these cells are known to secrete PSA. As shown in figure 3, our data clearly demonstrated that treatment of mice with sanguinarine (both pre- and post- tumor) resulted in an appreciable reduction in serum levels of prostate-specific antigen (PSA) in nude mice implanted with CWR22Rv1 cells. This is an important observation because serum PSA is considered to be an important marker for identifying humans CaP and, several investigators have also reported the usefulness of serum PSA as a follow up marker for local recurrence and/or distant disease in the patients after radical prostatectomy, radiation and hormonal therapy.

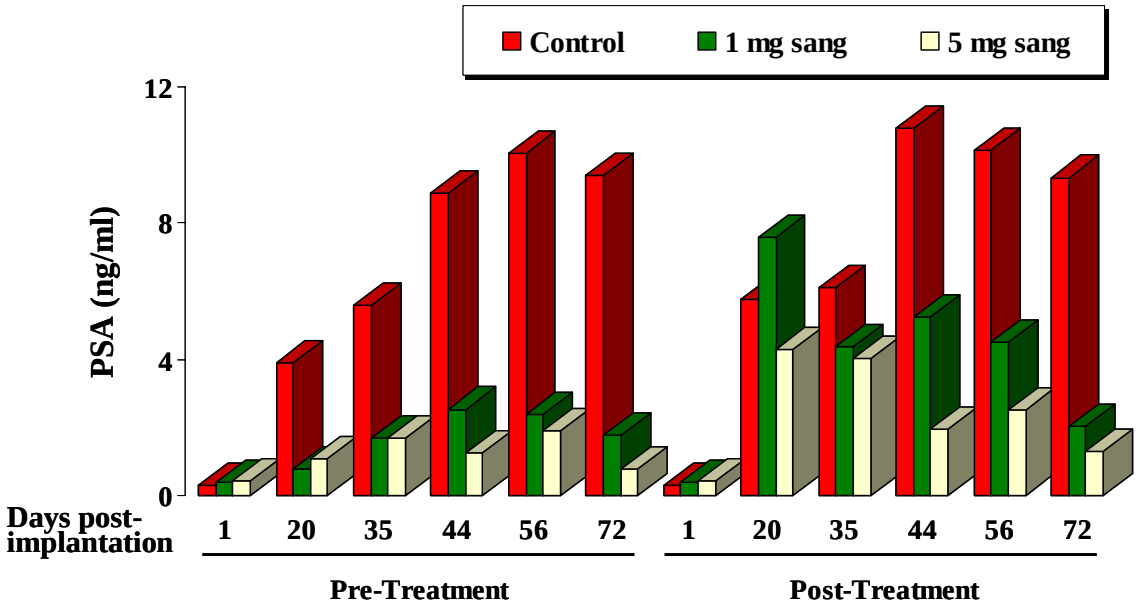


**Fig. 3: Effects of sanguinarine treatment on the levels of serum PSA in athymic nude mice implanted with CWR22Rv1 cells.** The cells (in matrigel) were implanted on both flanks of nude mice. The animals were treated with sanguinarine as described above. For determining PSA levels in the serum, following the treatments of animals with sanguinarine, at different times post-ctumor cell inoculation, blood was collected by 'madibular bleed' and serum was separated. The levels of PSA were determined by using a quantitative Human PSA enzyme linked immunosorbent assay (ELISA) kit (Anogen, Ontario, Canada) as per the manufacturer's protocol.

Thus, this study, for the first time, demonstrated the chemopreventive and therapeutic effects of sanguinarine against PCa development under in vivo situations. Based on our data, we suggest that sanguinarine is a promising candidate for chemoprevention and/or intervention against PCa.

**Effect of Sanguinarine on NF-κB Pathway:** Further, we also determined the mechanism of growth inhibitory effects of sanguinarine against CaP in nude mice implanted with CWR22Rv1 tumors. As shown below in figure 4, we found that sanguinarine treatments (in both the protocols) resulted in an appreciable down-modulation in the protein levels of NF-κB/p65 (in the nucleus) suggesting that the observed effects of sanguinarine may be mediated via inhibition of NF-κB pathway.

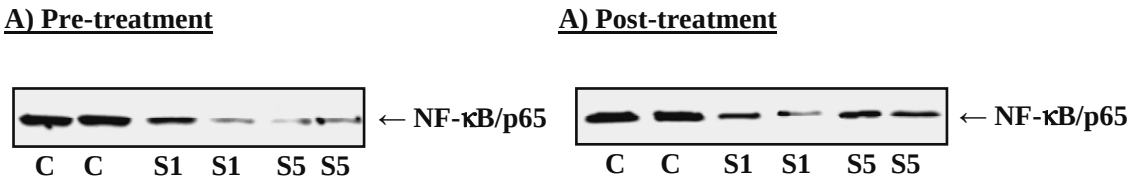


**Fig. 4: Effect of sanguinarine on nuclear levels of NF-κB/p65 in nude mice implanted with CWR22Rv1 tumors.** The cells (in matrigel) were implanted on both flanks of nude mice. The animals were treated with sanguinarine as described above. At the end of experiment, the mice were sacrificed and tumors were surgically removed and the levels of nuclear NF-κB/p65 were examined by the western blot analysis using the appropriate primary and secondary antibodies. The data shown here are representative of three independent immunoblots with similar results. **C = control; S1 = sanguinarine 1mg/ml; S5 = sanguinarine 5mg/ml.**

In addition, as shown below in figure 5, our data also demonstrated that sanguinarine treatment resulted in an appreciable decrease in the protein levels of anti-apoptotic Bcl-2 that is an established downstream target of NF-κB.

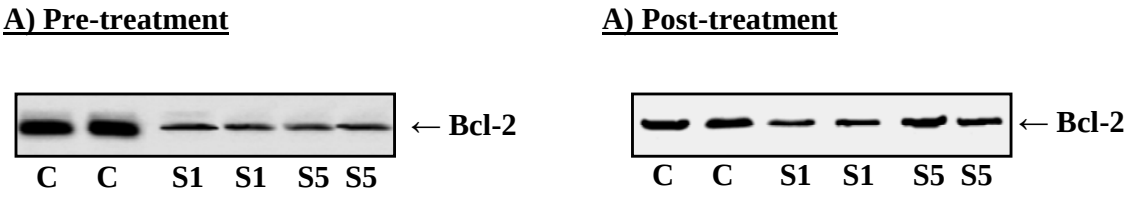


**Fig. 5: Effect of sanguinarine on Bcl-2 protein levels in nude mice implanted with CWR22Rv1 tumors.** The cells (in matrigel) were implanted on both flanks of nude mice. The animals were treated with sanguinarine as described above. At the end of experiment, the mice were sacrificed and tumors were surgically removed and the levels of Bcl-2 proteins were examined in total cell lysate with western blot analysis using the appropriate primary and secondary antibodies. The data shown here are representative of three independent immunoblots with similar results. **C = control; S1 = sanguinarine 1mg/ml; S5 = sanguinarine 5mg/ml.**

Furthermore, as shown in figure 6, our data also demonstrated that a down-modulation of cyclin D1 and cdk2 and an upregulation of WAF1/p21 during sanguinarine-mediated growth inhibition of CWR22Rv1-implanted prostate tumors in nude mice.

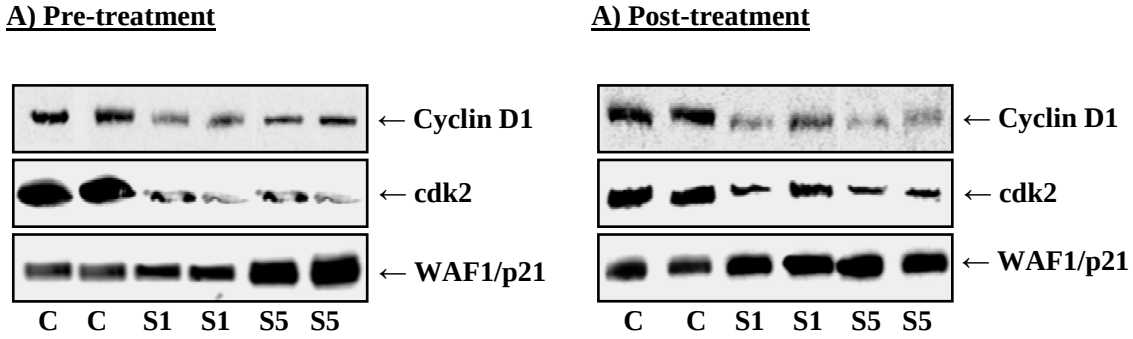


**Fig. 6: Effect of sanguinarine on cell cycle regulatory proteins in nude mice implanted with CWR22Rv1 tumors.** The cells (in matrigel) were implanted on both flanks of nude mice. The animals were treated with sanguinarine as described above. At the end of experiment, the mice were sacrificed and tumors were surgically removed and the levels of proteins were examined in total cell lysate with western blot analysis using the appropriate primary and secondary antibodies. The data shown here are representative of three independent immunoblots with similar results. **C = control; S1 = sanguinarine 1mg/ml; S5 = sanguinarine 5mg/ml.**

This study, for the first time, demonstrated the chemopreventive and therapeutic effects of sanguinarine against PCa development under *in vivo* situations. Based on our data, this can be suggested that sanguinarine is a promising candidate for chemoprevention and/or intervention against PCa.

***Evaluation of anti-proliferative effects of sanguinarine against prostate cancer in transgenic adenocarcinoma mouse prostate (TRAMP) model: A pilot study***

In order to assess the feasibility of our plan to determine the chemopreventive/therapeutic effects of sanguinarine against CaP in transgenic TRAMP mice that mimic the features of human disease, we first conducted a pilot study with limited number of animals.

***Study Design:***

In our pilot study, 12 male hetrerozygous C57BL/TGN TRAMP mice, line PB Tag 8247NG (12-14 weeks old; obtained from our breeding colony at the Animal Care Facility, School of Medicine, University of Wisconsin) were divided into three groups and subjected to sanguinarine treatments as indicated below:

**Group 1:** Control (PBS alone; *intraperitonial injection*; 5 days/week)
**Group 2:** Sanguinarine (1 mg/kg in PBS; *intraperitonial injection*; 5 days/week)
**Group 3:** Sanguinarine (5 mg/kg in PBS; *intraperitonial injection*; 5 days/week)

This treatment was given to the animals 5 days per week for 6 weeks. Throughout each experiment, the animals had free access to laboratory chow and water *ad libitum*. At the end of six weeks, the experiment was terminated and the animals from both experimental and control groups were sacrificed. At the time of sacrifice, the lower genitor-urinary (GU) tract, including the bladder, testes, seminal vesicles, and prostate, was removed en bloc. The GU wet weight was recorded followed by surgical dissection of the prostate gland. The weight of whole prostate gland was also recorded. Protein lysates were prepared from the prostate glands by homogenization in lysis buffer followed by the immunoblot analysis.

***Results & Conclusion:***

In this plot study with very limited number of animals, we found that compared to control, sanguinarine treatments resulted in an appreciable decrease in GU weight and prostate weight (data not shown). Further, the treatments were not found to have any evident toxic effects (body weight, food/fluid consumption) on the TRAMP mice (data not shown). Furthermore, we also assessed whether or not sanguinarine treatment affects the levels of transgene in TRAMP mice. As shown by the immunoblot analysis (Figure 7), treatment of mice with sanguinarine did not result in any significant change in the levels of transgene protein expression. This data clearly suggested that TRAMP mice are suitable model for our proposed pre-clinical trial.

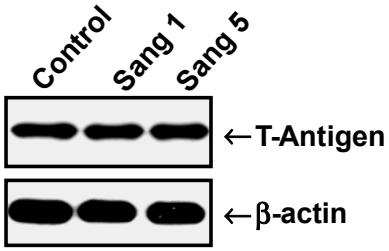


**Fig. 7: Effect of sanguinarine on protein levels of T-antigen in the prostate of TRAMP mice.** The effect of sanguinarine on the protein levels of T-antigen was assessed by immunoblot analyses using anti SV40TagAg antibody obtained from Santa Cruz Biotechnology., Inc. Equal loading was confirmed by reprobing the blot with β-actin. The data shown here are representative of three independent immunoblots with similar results.

***Evaluation of anti-proliferative effects of sanguinarine against prostate cancer in transgenic adenocarcinoma mouse prostate (TRAMP) model***

Following our pilot study described above, we conducted a detailed study to determine the anti-proliferative effects of sanguinarine against prostate cancer in TRAMP mice. In this regard, we conducted an

intervention study using TRAMP mice to assess the effect of oral supplementation of sanguinarine on tumor growth.

***Study Design:***

In this study, 26 male hetrerozygous C57BL/TGN TRAMP mice (Stock #3135, 8-15 weeks old; obtained from Jackson Labs) were divided into three groups and subjected to sanguinarine (obtained from Sigma Chemical CO., St Louis, MO) treatment as follows:

**Group 1:** Control (untreated)
**Group 2:** Sanguinarine (sanguinarine chloride in PBS; 1 mg/kg per mouse daily by *oral gavage*)
**Group 3:** Sanguinarine (sanguinarine chloride in PBS; 5 mg/kg per mouse daily by *oral gavage*).

This treatment was given to the animals 5 days per week starting at age 16 weeks until the mice were 32 weeks old or showed signs of distress. Throughout each experiment, the animals had free access to laboratory chow and water *ad libitum*. At the end of the 16 week treatment period, the experiment was terminated and the animals from both experimental and control groups were weighed then sacrificed. At the time of sacrifice, the lower GU tract, including the bladder, testes, seminal vesicles, and prostate, was removed en bloc. The GU wet weight was recorded followed by surgical dissection of the prostate gland. The weight of whole prostate gland was also recorded.

***Results & Conclusion:***

As shown by the data in figure 8, sanguinarine treatment groups showed a clear decreasing trend in prostate weight as well as total weight of the GU apparatus.

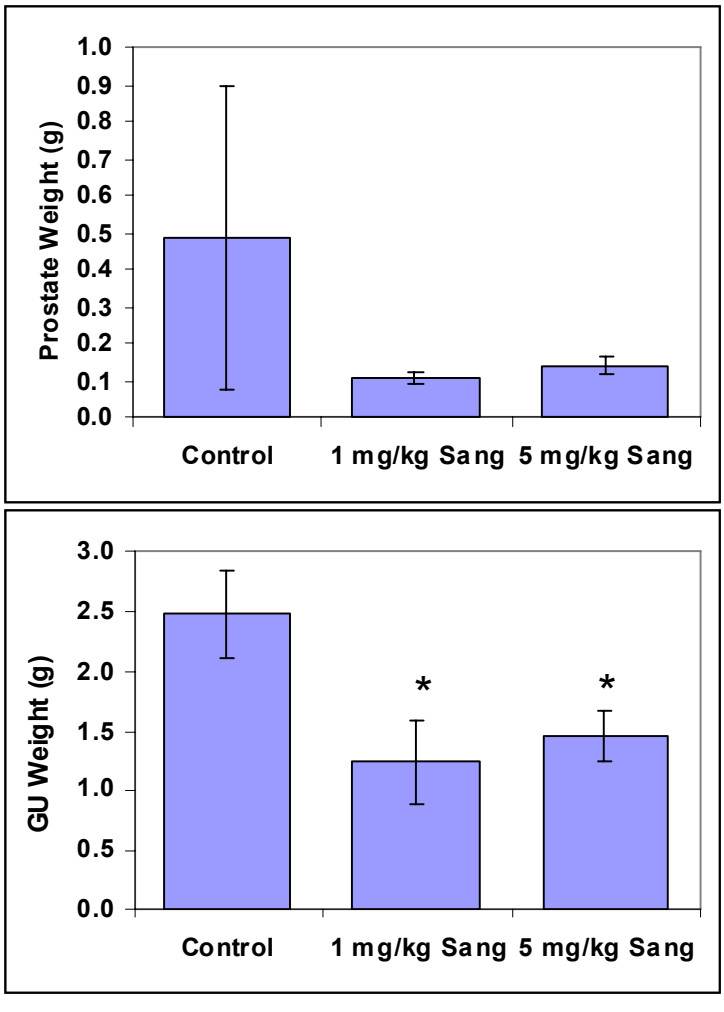


**Fig. 8: Effect of sanguinarine on the weights of prostate tumor and GU apparatus.** As described in the text, at the end of the study (32 weeks age of mice), the mice were sacrificed, the lower GU tract was dissected and weighed followed by dissection and weighing of the prostate tissue. * indicate a statistically significant difference compared with control mice with $p < 0.05$ with the T-test.

However, during dissections of the mice in our study, we noted abnormally large seminal vesicles concurrent with fewer and smaller prostate tumors in many mice. This was not consistent with earlier studies in our laboratory as well as reported by the other investigators. We discussed this issue with the Jackson Laboratories from where the mice were obtained. After a detailed investigation, the Jackson Laboratories confirmed problems with their breeding colony. This led Jackson Laboratories to start a new TRAMP colony from scratch. They have agreed to replace our mice in early 2008 for our studies. For this reason, we plan to repeat the experiments with new TRAMP mice, even when this funding from DOD is over. We will use departmental funds to carryout our remaining studies.

**Key Research Accomplishments:**

Based on our progress during the funding period, the key research accomplishments are itemized below.

1. *A major finding of this study is that sanguinarine causes cell cycle blockade and apoptosis of human CaP cells, irrespective of their androgen status. This is an important finding because CaP is known to undergo a transition from an early 'androgen-sensitive' form of cancer to a late (metastatic) 'androgen-insensitive' cancer, and at the time of clinical diagnosis most CaPs represent a mixture of androgen-sensitive and androgen-insensitive cells. Therefore, the key to the control of CaP appears to lie in the elimination of both types of prostate cancer cells (without affecting the normal cells) via mechanism-based preventive/therapeutic approaches. To our knowledge, this is the first study showing the involvement of cki-cyclin-cdk machinery during cell cycle arrest and apoptosis of CaP cells by sanguinarine.*

2. *We have demonstrated that sanguinarine possesses chemopreventive/anti-proliferative effects against prostate cancer in an athymic nude mice xenograft model. Further, our data suggested that sanguinarine-caused effects may be mediated via modulations in NF-κB pathway and cyclin kinase inhibitor-cyclin-cyclin dependent kinase machinery.*

3. *Our preclinical study with transgenic TRAMP model, which mimics human disease, has suggested that sanguinarine may be developed as an agent for chemoprevention/intervention of prostate cancer. However, more detailed studies are needed to support this assumption.*

**Reportable Outcome:**

The following two publications are directly associated with the funding from the DOD.


1. Adhami VM, Aziz MH, Reagan-Shaw SR, Nihal M, Mukhtar H, Ahmad N. Sanguinarine causes cell cycle blockade and apoptosis of human prostate carcinoma cells via modulation of cyclin kinase inhibitor-cyclin-cyclin-dependent kinase machinery. Mol Cancer Ther. 2004 Aug;3(8):933-40.

2. Aziz MH, Siddiqui IA, Ahsan H, Reagan-Shaw SR, Ahmad N. Preclinical evaluation of plant alkaloid sanguinarine against prostate cancer development in a nude mice xenograft model. Proc Amer Assoc Cancer Res 46: 1012-1013, 2005.


**Conclusions:**

In the 'Idea Development Award' selected for funding by the 'US Army Medical Research and Material Command', we proposed to test the hypothesis that ***a plant-derived alkaloid sanguinarine will impart antiproliferative effects against prostate cancer via a modulation in NF-κB-pathway-mediated apoptosis***. In

this grant, we proposed to validate our hypothesis in cell culture system as well as in animal models. Overall, we have made considerable progress with respect to our proposed hypothesis. However, unfortunately, our progress during this reporting period (01/16/2006 - 01/15/2007) was hampered due to several unforeseen circumstances. Because of this reason, a one-year no-cost extension of the grant was obtained in January 2007. Our experiment with TRAMP mice (from our breeding colony) could not be completed. Because of unexplainable reasons, the TRAMP mice died during the ongoing experiment to assess the chemopreventive effects of sanguinarine against CaP. Following this mishap, we purchased TRAMP mice from Jackson laboratory and conducted our proposed studies.

Our problems did not stop here because during dissections of the TRAMP mice in our main study, we noted abnormally phenotype (no or small prostate tumor and large seminal vesicles) in several TRAMP mice that was not consistent with earlier studies in our laboratory as well as reported by the other investigators. We discussed this issue with the Jackson Laboratories which confirmed problems with their breeding colony. This led Jackson Laboratories to start a new TRAMP colony from scratch. They have agreed to replace our mice in early 2008 for our studies. For this reason, we plan to repeat the experiments with new TRAMP mice, even when this funding from DOD is over. We will use departmental funds to carryout our remaining studies.

**References:**


1. Jemal, A., R. Siegel, E. Ward, T. Murray, J. Xu, and M. J. Thun: Cancer statistics, 2007. CA Cancer J. Clin. 57: 43-66, 2007.

2. Chaturvedi MM, Kumar A, Darnay BG, Chainy GBN, Agarwal S, Aggarwal BB: Sanguinarine (Pseudochelerythrine) is a potent inhibitor of NF-κB activation, IκBα phosphorylation, and degradation. J. Biol. Chem., 272: 30129-30134, 1997.

3. Walterova D, Ulrichova J, Valka I, Vicar J, Vavreckova C, Taborska E, Harjrader RJ, Meyer DL, Cerna H, Simanek V: Benzo[c]phenanthridine alkaloids sanguinarine and chelerythrine: biological activities and dental care applications. Acta Univ Palacki Olomuc Fac Med 139:7-16, 1995.

4. Mandel ID: Antimicrobial mouthrinses: overview and update. J Am Dent Assoc 125 Suppl 2: 2S-10S, 1994.

5. Adhami VM, Aziz MH, Mukhtar H, Ahmad N. Activation of prodeath Bcl-2 family proteins and mitochondrial apoptosis pathway by sanguinarine in immortalized human HaCaT keratinocytes. Clin Cancer Res 9: 3176-31782, 2003.

6. Ahmad N, Gupta S, Husain MM, Heiskanen KM, Mukhtar H. Differential antiproliferative and apoptotic response of sanguinarine for cancer cells versus normal cells. Clin Cancer Res 6:1524-1528, 2000.

7. Adhami VM, Aziz MH, Reagan-Shaw S, Nihal M, Mukhtar H and Ahmad N: Sanguinarine causes cell cycle blockade and apoptosis of human prostate carcinoma cells via modulation of cyclin kinase inhibitor-cyclin-cyclin dependent kinase machinery. Mol Cancer Ther 3: 933-940, 2004.

8. Reagan-Shaw S, Breur J, and Ahmad N: Enhancement of ultraviolet B radiation-mediated apoptosis by sanguinarine in HaCaT human immortalized keratinocytes. Mol Cancer Ther 5:418-429, 2006.

# Sanguinarine causes cell cycle blockade and apoptosis of human prostate carcinoma cells via modulation of cyclin kinase inhibitor-cyclin-cyclin-dependent kinase machinery

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## Abstract

**Prostate cancer is the second leading cause of cancer-related deaths in males in the United States. This warrants the development of novel mechanism-based strategies for the prevention and/or treatment of prostate cancer. Several studies have shown that plant-derived alkaloids possess remarkable anticancer effects. Sanguinarine, an alkaloid derived from the bloodroot plant *Sanguinaria canadensis*, has been shown to possess antimicrobial, anti-inflammatory, and antioxidant properties. Previously, we have shown that sanguinarine possesses strong antiproliferative and proapoptotic properties against human epidermoid carcinoma A431 cells and immortalized human HaCaT keratinocytes. Here, employing androgen-responsive human prostate carcinoma LNCaP cells and androgen-unresponsive human prostate carcinoma DU145 cells, we studied the antiproliferative properties of sanguinarine against prostate cancer. Sanguinarine (0.1–2 μmol/L) treatment of LNCaP and DU145 cells for 24 hours resulted in dose-dependent (1) inhibition of cell growth [as evaluated by 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide assay], (2) arrest of cells in $G_0$-$G_1$ phase of the cell cycle (as assessed by DNA cell cycle analysis), and (3) induction of apoptosis (as evaluated by DNA ladder formation and flow cytometry). To define the mechanism of antiproliferative effects of sanguinarine against prostate cancer, we studied the effect of sanguinarine on critical molecular events known to regulate the cell cycle and the apoptotic machinery. Immunoblot analysis showed that sanguinarine treatment of both LNCaP and DU145 cells resulted in significant (1) induction of cyclin kinase inhibitors p21/WAF1 and p27/KIP1; (2) down-regulation of cyclin E, D1, and D2; and (3) down-regulation of cyclin-dependent kinase 2, 4, and 6. A highlight of this study was the fact that sanguinarine induced growth inhibitory and antiproliferative effects in human prostate carcinoma cells irrespective of their androgen status. To our knowledge, this is the first study showing the involvement of cyclin kinase inhibitor-cyclin-cyclin-dependent kinase machinery during cell cycle arrest and apoptosis of prostate cancer cells by sanguinarine. These results suggest that sanguinarine may be developed as an agent for the management of prostate cancer. [Mol Cancer Ther 2004;3(8):933–40]**

## Introduction

Prostate cancer is a common malignancy and, next only to lung cancer, is the second leading cause of cancer-related deaths of males in the United States (1). According to an estimate of the American Cancer Society, a total of 230,110 men will be diagnosed with prostate cancer in the United States in the year 2004 and 29,900 prostate cancer-related deaths are predicted for 2004 (1). The major cause of mortality from this disease is metastasis of hormone-refractory cancer cells that fail to respond to hormone ablation therapy (2, 3). Because surgery and current treatment options have proven to be inadequate in curing or controlling prostate cancer, the search for novel agents for the management of this disease has become a priority. In the recent past, agents obtained from herbs and plants have gained considerable attention for the prevention and/or treatment of certain cancer types including prostate cancer (4).

Naturally occurring plant-based agents often provide opportunities for the management of cancer and other diseases (ref. 5 and references therein). Sanguinarine (13-methyl[1,3]benzodioxolo[5,6-*c*]-1,3-dioxolo[4,5]phenanthridinium; Fig. 1), derived from the root of *Sanguinaria canadensis* and other poppy-fumaria species, is a benzophenanthridine alkaloid and a structural homologue of chelerythrine. It has been shown to possess antimicrobial, antioxidant, anti-inflammatory, and antitumor properties (6). It is widely used in toothpaste and mouthwash for the prevention/treatment of gingivitis and other inflammatory conditions (7–9). There is a suggestion for the antitumor properties of this alkaloid (6, 10–16). In a recent study, we have shown that sanguinarine, at micromolar concentrations, imparts cell growth inhibitory responses in human squamous carcinoma (A431) cells via an induction of apoptosis (10). The important observation of this

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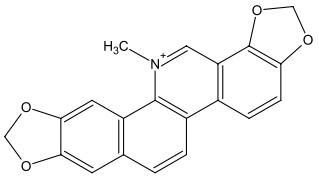

**Figure 1.** Chemical structure of sanguinarine (13-methyl[1,3]benzodioxolo[5,6-*c*]-1,3-dioxolo[4,5]phenanthridinium).

study was that sanguinarine treatment did not result in apoptosis of the normal human epidermal keratinocytes at similar dose (10). In another recent study, we showed that sanguinarine causes an apoptotic death of immortalized human keratinocytes (HaCaT) via modulations in the mitochondrial pathway and the Bcl-2 family of proteins (11). The present work is our mechanism-based effort to assess if sanguinarine could be developed as an agent for the management of prostate cancer. We assessed the antiproliferative effects of sanguinarine on growth/proliferation of human prostate cancer cells and the involvement of cell cycle regulatory events as the mechanism of this response.

Uncontrolled cellular proliferation is a hallmark of all cancers, and the blockade of the cell cycle is regarded as an effective strategy for eliminating cancer cells (17–23). In fact, at present, various cell cycle inhibitors are being evaluated as therapeutic tools for the management of cancer in preclinical and clinical studies. The cell cycle in eukaryotes is controlled (at least in part) by a family of protein kinase complexes wherein each complex is composed of a catalytic subunit, the cyclin-dependent kinase (cdk), and its essential regulatory subunit, the cyclin (24–27). These complexes are activated at specific intervals during the cell cycle and can also be regulated by exogenous factors (26). The cyclin-cdk complexes are subject to inhibition via binding to a class of proteins known as the cyclin kinase inhibitors (cki). Anticancer agents may alter one or more regulatory events in the cell cycle resulting in blockade of cell cycle progression, thereby reducing the growth and proliferation of the cancer cells. Cell cycle blockade may ultimately lead to a programmed death (i.e., apoptosis of cancer cells). The ability of tumor cells to evade apoptosis plays a significant role in their resistance to conventional therapeutic regimens (28). Therefore, search for novel agents designed to impart cell cycle arrest and induction of apoptosis in cancer cells is being earnestly pursued.

In the present study, we show that sanguinarine imparts antiproliferative effects against androgen-responsive (LNCaP) and androgen-unresponsive (DU145) human prostate cancer cells and that this effect is mediated through dysregulation of cell cycle and induction of apoptosis. To our knowledge, this is the first study showing the modulation of cell cycle regulatory events by sanguinarine.

## Materials and Methods

### Reagents

Sanguinarine (>98% pure) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were obtained from Sigma Chemical Co. (St. Louis, MO). The antibodies (p21; p27; cyclin E, D1, and D2; and cdk 2, 4, and 6) used in this study were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Apo-direct apoptosis kit was obtained from Phoenix Flow Systems (San Diego, CA). The DC protein assay kit was obtained from Bio-Rad Laboratories (Hercules, CA). Novex precast Tris-glycine gels were obtained from Invitrogen (Carlsbad, CA).

### Cell Culture

The androgen-responsive human prostate carcinoma cells LNCaP and androgen-unresponsive human prostate carcinoma cells DU145 were obtained from American Type Culture Collection (Rockville, MD). DU145 cells were cultured in MEM with 2 mmol/L L-glutamine and Eagle's balanced salt solution adjusted to contain 1.5 g/L sodium bicarbonate, 0.1 mmol/L nonessential amino acids, 1.0 mmol/L sodium pyruvate, 10% fetal bovine serum, and 1% penicillin-streptomycin. LNCaP cells were cultured in RPMI 1640 (Life Technologies, Inc., Grand Island, NY) with 2 mmol/L L-glutamine adjusted to contain 1.5 g/L sodium bicarbonate, 4.5 g/L glucose, 10 mmol/L HEPES, 1.0 mmol/L sodium pyruvate, 10% fetal bovine serum, and 1% penicillin-streptomycin. The cells were maintained under standard cell culture conditions at 37°C and 5% $CO_2$ in a humid environment.

### Treatment of Cells

Sanguinarine (dissolved in ethanol) was employed for the treatment of cells. The cells (70% to 80% confluent) were treated with sanguinarine (0.1, 0.2, 0.5, 1, and 2 μmol/L) for 24 hours in complete cell culture medium. Cells that were used as controls were incubated with the maximum used amount of ethanol only.

### Cell Growth/Cell Viability

The effect of sanguinarine on the viability of cells was determined by MTT assay. The cells were plated at $2 \times 10^5$ cells per well in 200 μL DMEM containing 0.1, 0.2, 0.5, 1, and 2 μmol/L sanguinarine in a 96-well microtiter plate. Each concentration of sanguinarine was repeated in 10 wells. The cells were incubated for 24 hours at 37°C in a humidified chamber. Following 24 hours of incubation, MTT reagent (4 μL, 5 mg/mL in PBS) was added to each well and incubated for 2 hours. The microtiter plate containing the cells was centrifuged at 1,800 rpm for 5 minutes at 4°C. The MTT solution was removed from the wells by aspiration and the formazan crystals were dissolved in DMSO (150 μL). Absorbance was recorded on a microplate reader at 540 nm wavelength. The effect of sanguinarine on growth inhibition was assessed as percentage inhibition in cell growth wherein vehicle-treated cells were taken as 100%.

### Detection of Apoptosis by DNA Ladder Assay

The LNCaP and DU145 cells were grown to ~70% confluency and treated with sanguinarine (0.1, 0.2, 0.5, 1, and 2 μmol/L) for 24 hours. Following this treatment, the

cells were washed twice with PBS [10 mmol/L Tris (pH 7.5), 150 mmol/L NaCl, 5 mmol/L $MgC1_2$, 0.5% Triton X-100], left on ice for 15 minutes, and pelleted by centrifugation (14,000 × *g*) at 4°C. The pellet was incubated with nuclear lysis buffer [10 mmol/L Tris (pH 7.5), 400 mmol/L NaCl, 1 mmol/L EDTA, 1% Triton X-100] for 30 minutes on ice and centrifuged at 14,000 × *g* at 4°C. The supernatant obtained was incubated overnight with RNase (0.2 mg/mL) at room temperature and with proteinase K (0.1 mg/mL) for 2 hours at 37°C. DNA was extracted using phenol/chloroform (1:1) and precipitated with 95% ethanol for 2 hours at −80°C. The DNA precipitate was centrifuged at 14,000 × *g* at 4°C for 15 minutes, and the pellet was air dried and dissolved in Tris-EDTA buffer [20 μL, 10 mmol/L Tris-HCl (pH 8.0), 1 mmol/L EDTA]. Total amount of DNA was resolved over 1.5% agarose gel containing 0.3 μg/mL ethidium bromide in 1× Tris-borate EDTA buffer (pH 8.3; 89 mmol/L Tris, 89 mmol/L boric acid, 2 mmol/L EDTA; BioWhittaker, Inc., Walkersville, MD). The bands were visualized under UV transilluminator (Model TM-36, UVP Inc., San Gabriel, CA) followed by Polaroid photography (MP-4 Photographic System, Fotodyne Inc., Hartland, WI).

### Quantitiation of Apoptosis by Flow Cytometry

The cells were grown at a density of $1 \times 10^6$ cells in 100 mm culture dishes and treated with sanguinarine (0.1, 0.2, 0.5, 1, and 2 μmol/L) for 24 hours. The cells were trypsinized, washed with PBS, and processed for labeling with fluorescein-tagged dUTP nucleotide and propidium iodide using Apo-direct apoptosis kit (Phoenix Flow Systems) as per the manufacturer's protocol. Labeled cells were analyzed by flow cytometry.

### DNA Cell Cycle Analysis

The cells (70% confluent) were treated with sanguinarine (0.1, 0.2, 0.5, 1, and 2 μmol/L) in complete medium for 24 hours. The cells were trypsinized thereafter, washed twice with cold PBS, and centrifuged. The cell pellet was resuspended in 50 μL cold PBS to which cold methanol (450 μL) was added and the cells were incubated for 1 hour at 4°C. The cells were centrifuged at 1,100 rpm for 5 minutes, pellet washed twice with cold PBS, suspended in 500 μL PBS, and incubated with 5 μL RNase (20 μg/mL final concentration) for 30 minutes. The cells were chilled over ice for 10 minutes and incubated with propidium iodide (50 μg/mL final concentration) for 1 hour and analyzed by flow cytometry.

### Preparation of Cell Lysates and Western Blot Analysis

The cells were harvested at 24 hours following sanguinarine treatment as described above and washed with cold PBS (10 mmol/L, pH 7.4). The cells were incubated in ice-cold lysis buffer [50 mmol/L Tris-HCl, 150 mmol/L NaCl, 1 mmol/L EGTA, 1 mmol/L EDTA, 20 mmol/L NaF, 100 mmol/L $Na_3VO_4$, 0.5% NP40, 1% Triton X-100, 1 mmol/L phenylmethylsulfonyl fluoride (pH 7.4)] with freshly added protease inhibitor cocktail (Protease Inhibitor Cocktail Set III, Calbiochem, La Jolla, CA) over ice for 30 minutes. The cells were scraped and the lysate was collected in a microfuge tube and passed through a 21.5 G needle to break up the cell aggregates. The lysate was cleared by centrifugation at 14,000 × *g* for 15 minutes at 4°C, and the supernatant (total cell lysate) was collected, aliquoted, and stored at −70°C. The protein content in the lysates was measured by DC protein assay (Bio-Rad Laboratories) as per the manufacturer's protocol.

For Western blot analysis, protein (20–50 μg) was resolved over 8% to 12% SDS-PAGE gels and transferred onto a nitrocellulose membrane. The nonspecific sites were blocked by incubating the blot with 5% nonfat dry milk in buffer (containing 10 mmol/L Tris, 100 mmol/L NaCl, 0.1% Tween 20) for 1 hour at room temperature or overnight at 4°C. The blot was washed with wash buffer (10 mmol/L Tris, 100 mmol/L NaCl, 0.1% Tween 20) for 2 × 10 minutes and incubated overnight with appropriate primary antibody specific for the protein to be assessed. The antibodies were used at dilutions specified by the manufacturer. The blot was washed for 2 × 10 minutes followed by an incubation with the corresponding secondary antibody horseradish peroxidase conjugate (Amersham Life Science, Inc., Arlington Heights, IL) at 1:2,000 dilution for 1 hour at room temperature. The blot was washed in wash buffer twice for 10 minutes each and four times for 5 minutes each. The protein was detected by chemiluminescence using enhanced chemiluminescence kit (Amersham Life Science) and autoradiography with XAR-5 film (Amersham Life Science). For every immunoblot, equal loading of protein was confirmed by stripping the blot and reprobing with β-actin antibody.

### Statistical Analysis

Results were analyzed using a two-tailed Student's *t* test to assess statistical significance. Values of $P < 0.05$ were considered statistically significant.

## Results and Discussion

Prostate cancer in humans progresses from an androgen-responsive to an androgen-unresponsive state, and at the time of clinical diagnosis, most prostate cancers represent a mixture of androgen-responsive and androgen-unresponsive cells (29). Whereas androgen-responsive cells undergo rapid apoptosis on androgen ablation, androgen-unresponsive cells evade apoptosis during androgen withdrawal, although they retain the molecular machinery for apoptosis. Mortality from prostate cancer generally occurs from the proliferation and invasion of these androgen-unresponsive cells, which fail to undergo apoptosis culminating into hormone-refractory prostate cancer for which no cure but only palliative treatment is available (3). Therefore, there is an urgent need to intensify our efforts for a better understanding of this disease and for the development of novel mechanism-based approaches for its prevention and treatment (30).

Earlier studies in cell culture system from our laboratory showed that sanguinarine treatment resulted in an apoptotic death of A431 carcinoma cells (10). In fact, this report was the first systematic study showing the anticancer effect of sanguinarine. In the present study, we assessed the

anticancer effects of this plant-based alkaloid against prostate cancer. For this study, we employed two human prostate cancer cell lines DU145 and LNCaP. The choice of these two cells lines was based on the fact that LNCaP cells are androgen responsive and DU145 cells are androgen unresponsive and that, at the time of clinical diagnosis, most prostate cancers present as a mixture of androgen-responsive and androgen-unresponsive cells. Therefore, eliminating both cell types seems to be an effective approach for the management of prostate cancer. In the first set of experiments, we evaluated whether sanguinarine treatment imparts antiproliferative effects in human prostate cancer cells. Employing the MTT assay, we observed that sanguinarine (0.1–2 μmol/L) treatment of DU145 and LNCaP cells resulted in dose-dependent decrease in the growth of both cell types (Fig. 2). Interestingly, an $IC_{50}$ of ~1 μmol/L was observed for both cell types. Sanguinarine has been shown to induce apoptosis in certain types of cancer and transformed cells (10–16). Studies have shown that, at low concentrations, sanguinarine treatment of cancer cells induced apoptosis distinguished by cell surface blebbing whereas, at higher concentrations, sanguinarine caused a second mode of cell death, oncosis, distinguished by cell surface blistering (13–16). In this study, we determined if the observed growth inhibition of LNCaP and DU145 cells by low concentrations of sanguinarine is mediated via apoptosis. As shown in Fig. 3, our data showed that sanguinarine

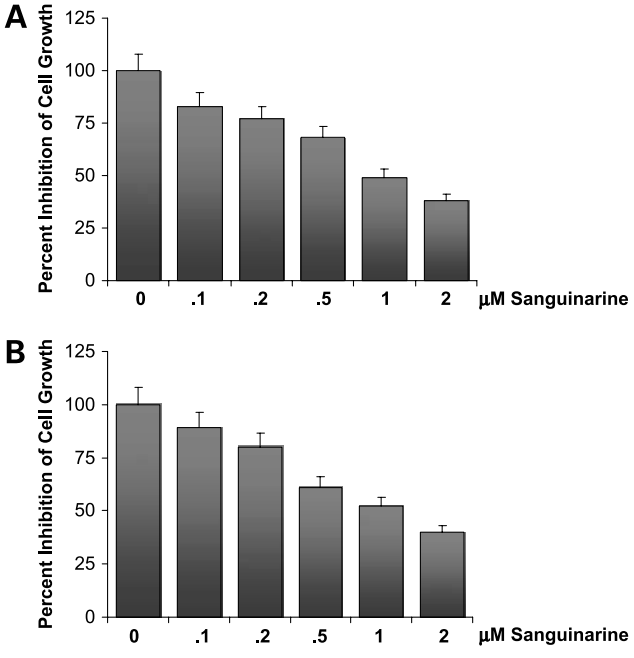


**Figure 2.** Effect of sanguinarine on the growth of prostate cancer cells LNCaP (**A**) and DU145 (**B**). Cells were treated with sanguinarine (0.1, 0.2, 0.5, 1, and 2 μmol/L), and the percentage inhibition of cell growth was determined by MTT assay in a 96-well ELISA plate as detailed in Materials and Methods. *Columns,* mean of three separate experiments wherein each treatment was repeated in 10 wells; *bars,* SE.

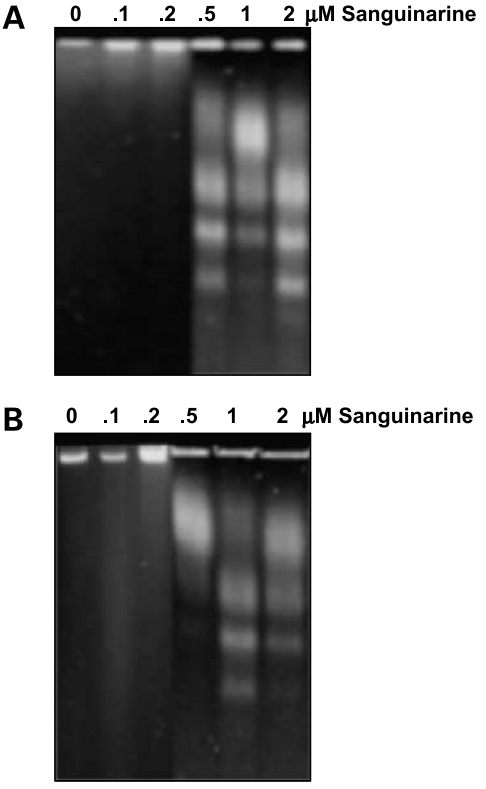


**Figure 3.** Effect of sanguinarine on DNA fragmentation in prostate cancer cells LNCaP (**A**) and DU145 (**B**) as analyzed by DNA ladder formation. Cells were grown to 70% confluency and treated with sanguinarine (0.1, 0.2, 0.5, 1, and 2 μmol/L) for 24 hours. The DNA was isolated and resolved over 1.5% agarose gel followed by visualization of bands as described in Materials and Methods. Data are representative of an experiment repeated three times with similar results.

**Table 1. Effect of sanguinarine on apoptosis in prostate cancer cells LNCaP and DU145 as analyzed by flow cytometry**

| Treatment | % Apoptosis | |
|---|---|---|
| | LNCaP Cells | DU145 Cells |
| Control | 2.7 ± 0.03 | 3.1 ± 0.02 |
| Sanguinarine (μmol/L) | | |
| 0.1 | 4.4 ± 0.25 | 5.3 ± 0.36 |
| 0.2 | 6.1 ± 0.08 | 5.9 ± 0.08 |
| 0.5 | 12.2 ± 0.38* | 13.2 ± 0.68* |
| 1.0 | 19.0 ± 2.34† | 18.6 ± 3.11† |
| 2.0 | 41.1 ± 2.47‡ | 43.7 ± 4.13‡ |

Cells were treated with sanguinarine (0.1, 0.2, 0.5, 1, and 2 μmol/L) for 24 hours and labeled with dUTP using terminal deoxynucleotidyl transferase and propidium iodide. Cells showing dUTP fluorescence above that of the control population are considered as apoptotic and their percentage population is shown. Data are means ± SE of three experiments done in triplicate.
*$P < 0.05$.
†$P < 0.01$.
‡$P < 0.001$.

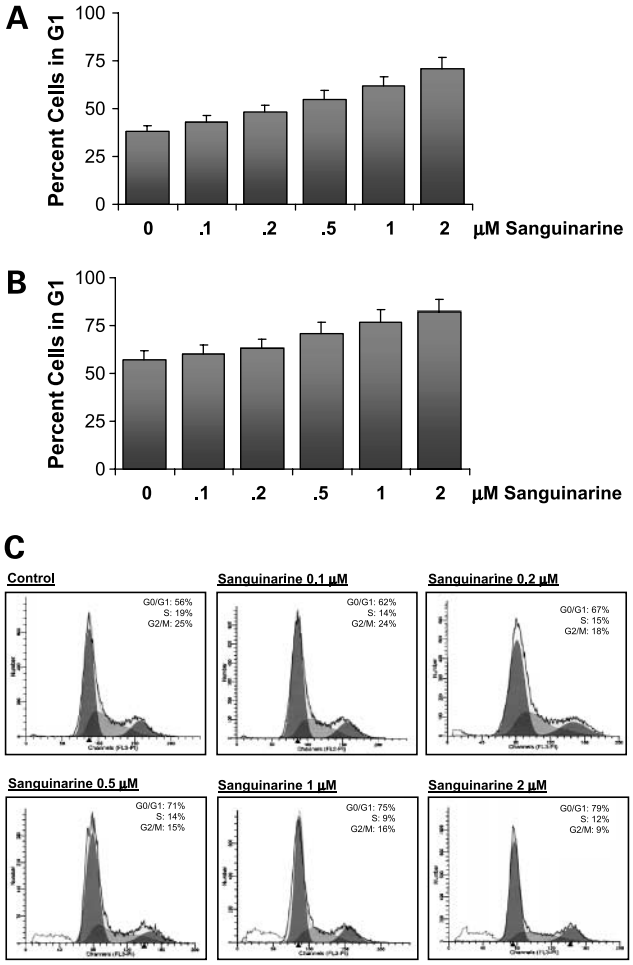


**Figure 4.** Effect of sanguinarine on cell cycle in prostate cancer cells. The growing cells (~60% confluent) were treated with sanguinarine (0.1, 0.2, 0.5, 1, and 2 μmol/L) for 24 hours, and the DNA cell cycle analysis was done as described in Materials and Methods. *Columns,* mean of three separate experiments conducted in triplicate with LNCaP cells (**A**) and DU145 cells (**B**); *bars,* SE. **C,** data are representative of actual analysis of cell cycle distribution for DU145 cells done in triplicate with similar results.

treatment of both androgen-responsive LNCaP cells and androgen-unresponsive DU145 cells resulted in the formation of DNA ladder, a hallmark of apoptosis. These results were further verified by terminal deoxynucleotidyl transferase–mediated nick end labeling assay.

As shown by the data in Table 1, sanguinarine treatment to both cell lines resulted in a dose-dependent increase in terminal deoxynucleotidyl transferase–mediated nick end labeling positive (apoptotic) cells. Apoptotic cell death is the consequence of a series of precisely regulated events that are frequently altered in tumor cells. This provides an opportunity for selective clinical intervention to induce a programmed death of the cancer cells, ideally without affecting the normal cells (28). Apoptosis is a physiologic process that involves elimination of cells with DNA damage (31) and represents a distinct form of cell death that differs from necrotic cell death (32). Hence, agents that can modulate apoptosis may be useful in the management and therapy of cancer (33, 34).

Several studies have shown that the induction of apoptosis may be cell cycle dependent (refs. 35–39 and references therein). Therefore, in our next series of experiments, we tested the hypothesis that sanguinarine-caused apoptosis of LNCaP and DU145 cells is mediated via cell cycle blockade. We therefore did DNA cell cycle analysis to assess the effect of sanguinarine treatment on the distribution of cells in the cell cycle. As shown in Fig. 4, compared with vehicle treatment, sanguinarine treatment was found to result in dose-dependent accumulation of DU145 cells in $G_1$ phase of the cell cycle. Similar results were observed when LNCaP cells were treated with increasing dose of sanguinarine (Fig. 4). This observation is important because the molecular analyses of human cancers have revealed that cell cycle regulators are frequently mutated in most common malignancies (40, 41). Therefore, in recent years, inhibition of the cell cycle has been appreciated as a target for the management of cancer (42, 43).

We next studied the involvement of cki-cyclin-cdk machinery in $G_1$-phase cell cycle arrest of human prostate cancer cells by sanguinarine. The journey of cells through the cell cycle in eukaryotes is coordinated by a family of

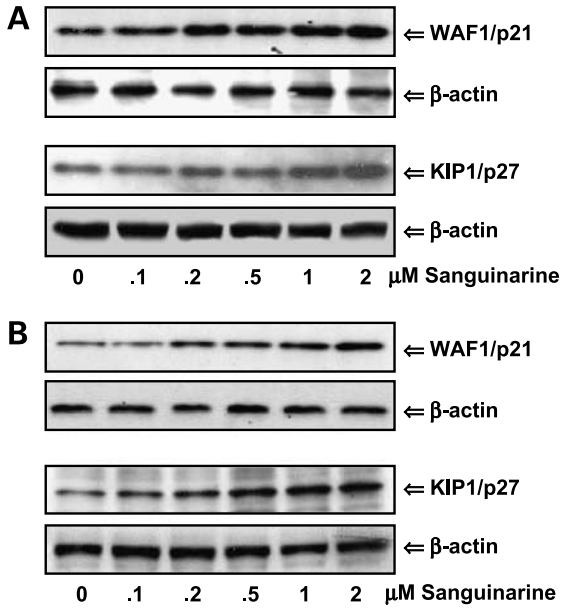


**Figure 5.** Effect of sanguinarine on the protein expression of p21/WAF1 and p27/KIP1 in prostate cancer cells LNCaP (**A**) and DU145 (**B**). The cells were treated with sanguinarine (0.1, 0.2, 0.5, 1, and 2 μmol/L) and harvested at 24 hours following the treatment. Total cell lysates were prepared and protein (50 μg) was subjected to SDS-PAGE followed by Western blot analysis using specific antibodies and secondary horseradish peroxidase – conjugated antibodies. The protein was detected by chemiluminescence. Details are described in Materials and Methods. Equal loading was confirmed by stripping the membrane and reprobing it with β-actin. Data are representative of a typical experiment repeated three times with similar results.

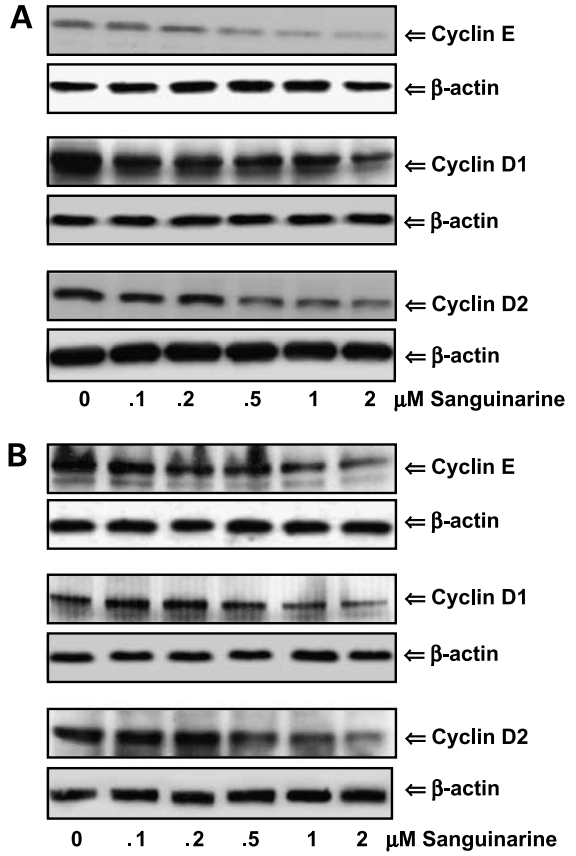


**Figure 6.** Effect of sanguinarine on the protein expression of cyclin E, D1, and D2 in prostate cancer cells LNCaP (**A**) and DU145 (**B**). The cells were treated with sanguinarine (0.1, 0.2, 0.5, 1, and 2 μmol/L) and harvested at 24 hours following the treatment. Total cell lysates were prepared and protein (20 μg) was subjected to SDS-PAGE followed by Western blot analysis using specific primary antibodies and secondary horseradish peroxidase–conjugated antibodies. The proteins were detected by chemiluminescence. Equal loading was confirmed by stripping the membrane and reprobing it with β-actin. Details are described in Materials and Methods. Data are representative of a typical experiment repeated three times with similar results.

protein kinase complexes. Each complex is composed minimally of cyclins (regulatory subunit) that bind to cdks (catalytic subunit) to form active cyclin-cdk complexes. These complexes are activated at various checkpoints after specific intervals during the cell cycle and can also be regulated by several exogenous factors (40). However, in transformed cells, cell cycle progression could be a mitogenic signal-dependent or mitogenic signal-independent process (44, 45). Cdk activity is additionally regulated by small proteins known as ckis. Ckis include the p21/WAF1 and p27/KIP1 family of proteins. Therefore, we studied the modulation in cell cycle regulatory events operative in the $G_0$-$G_1$ phase as a mechanism of sanguinarine-mediated cell cycle dysregulation and apoptosis in human prostate cancer cells. As shown by Western blot analysis (Fig. 5A), sanguinarine treatment (0.2–2.0 μmol/L for 24 hours) of LNCaP cells resulted in significant dose-dependent up-regulation of the ckis p21/WAF1 and p27/KIP1. Interestingly, similar results were obtained with DU145 cells (Fig. 5B). Many studies have shown that these ckis regulate the progression of cells in the $G_0$-$G_1$ phase of the cell cycle, and an induction of these molecules causes a blockade of $G_1$-S transition, thereby resulting in a $G_0$-$G_1$ phase arrest (46). Further, studies have also shown that loss of functional cki in different human cancers and derived cell lines leads to uncontrolled cell proliferation due to an increase in the levels of cdk-cyclin complex (47). p21/WAF1/CIP is an important cki and is shown to be almost a universal inhibitor of cdks (48, 49). Many studies have shown that certain exogenous stimuli may result in a p53-dependent and p53-independent induction of p21/WAF1, which in turn may trigger a series of events, ultimately resulting in a cell cycle arrest and/or apoptosis

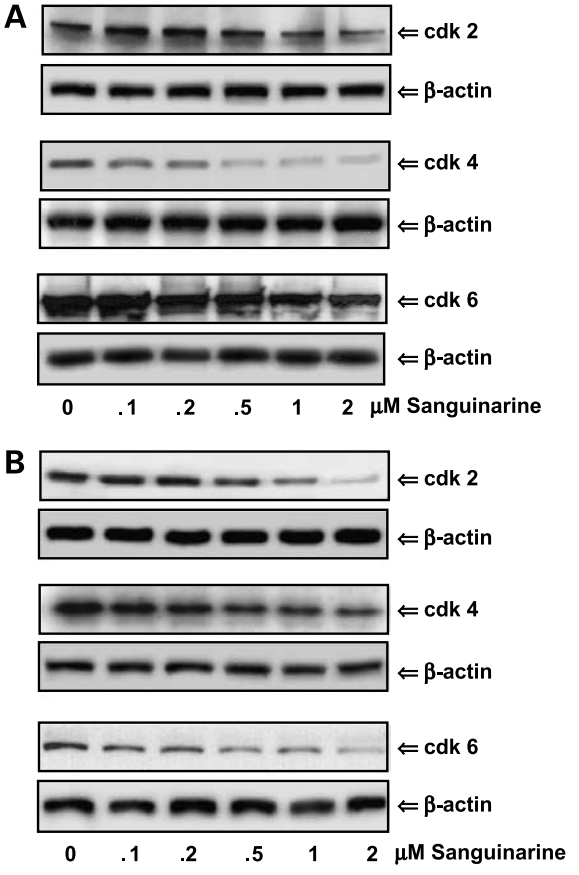


**Figure 7.** Effect of sanguinarine on the protein expression of cdk 2, 4, and 6 in prostate cancer cells LNCaP (**A**) and DU145 (**B**). The cells were treated with sanguinarine (0.1, 0.2, 0.5, 1, and 2 μmol/L) and harvested at 24 hours following the treatment. Total cell lysates were prepared and protein (20 μg) was subjected to SDS-PAGE followed by Western blot analysis using appropriate primary antibodies and secondary horseradish peroxidase–conjugated antibodies. The proteins were detected by chemiluminescence. Equal loading was confirmed by stripping the membrane and reprobing it with β-actin. Details are described in Materials and Methods. Data are representative of a typical experiment repeated twice with similar results.

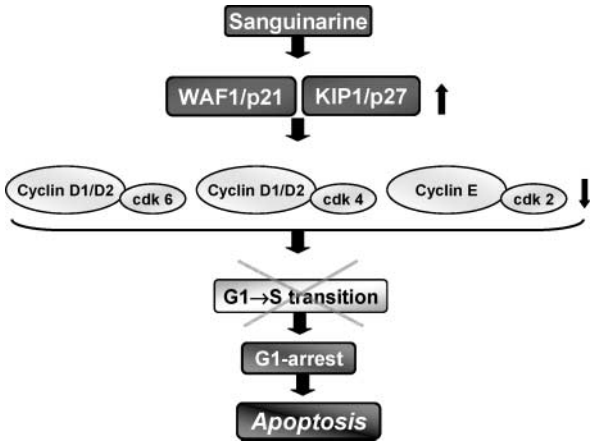


**Figure 8.** Proposed model for sanguinarine-mediated cell cycle arrest and apoptosis of cancer cells.

(46–49). Our data showing an induction of p21/WAF1 by sanguinarine seem to be p53 dependent in LNCaP cells (with wild-type p53) because sanguinarine treatment to these cells was found to result in a concentration-dependent increase in protein levels of p53 (data not shown). Further, the induction of p21/WAF1 by sanguinarine seems to be p53 independent in DU145 cells (with mutant p53). Thus, sanguinarine seems to impart its growth inhibitory and cell cycle dysregulatory effects irrespective of p53 status. However, more studies are required to assess a definite association between p53 status in cancer cells and the biological effects of sanguinarine. Recent studies (46, 50) have shown the critical role of p27/KIP1 in apoptosis and cell cycle progression through $G_0$-$G_1$ phase. We observed a significant induction of p27/KIP1 by sanguinarine. Thus, sanguinarine imparts cell cycle dysregulation in both androgen-sensitive and androgen-insensitive human prostate carcinoma cells via an up-regulation of ckis involved in $G_0$-$G_1$ progression.

Several studies have shown that overexpression of cyclins and cdks is commonly associated with human malignancies (47, 51). Therefore, we next evaluated the effect of sanguinarine treatment on modulations in the levels of the major cyclins operative in $G_0$-$G_1$ phase of the cell cycle, viz., cyclin D1, D2, and E. We observed that treatment of LNCaP and DU145 cells with sanguinarine (0.2–2.0 μmol/L for 24 hours) resulted in a dose-dependent decrease in the protein expression of cyclin D1, D2, and E in both cell types (Fig. 6). Similarly, we found that treatment of LNCaP and DU145 cells with sanguinarine (0.2–2.0 μmol/L) for 24 hours resulted in a dose-dependent decrease in cdk 2, 4, and 6 in LNCaP and DU145 cells (Fig. 7). Studies have shown that cdk 2, 4, and 6 are critical for progression of cells through $G_1$ and entry into the S phase of the cell cycle (47, 52). These results suggest that sanguinarine is capable of restoring proper checkpoint control in both types of human prostate carcinoma cells.

Cell cycle regulatory molecules are the critical regulatory elements, which control the progression of cells in early and late $G_1$ phases of the cell cycle (46–53). Our data, showing a decrease in the protein levels of the cyclin D1, D2, and E and cdk 2, 4, and 6 by sanguinarine treatment in both cell lines, agree with the fact that the cdks and cyclins operate in association with each other by forming complexes, which may bind to and are inhibited by ckis. This series of events imposes a blockade of $G_1$-S transition, resulting in $G_0$-$G_1$ phase arrest of the cell cycle. Thus, taken together, as shown in the composite scheme in Fig. 8, we suggest the series of events by which sanguinarine results in the blockade of cell cycle via imposing an artificial checkpoint at $G_1$-S transition. This causes an arrest of cancer cells in the $G_1$ phase of the cell cycle, which is an irreversible process that ultimately results in an apoptotic cell death. Several other possibilities of cell cycle arrest by sanguinarine cannot be ruled out. It is also possible that the down-regulation of cyclin D/cdk4/cdk6 is the cause for cell cycle arrest, whereas the modulations in the levels of p21/WAF1 and p27/KIP1 by sanguinarine are regulated with completely different mechanisms such as at a transcriptional level via p53-dependent and p53-independent pathways (in case of p21/WAF1) and through post-translational mechanisms such as proteasome-mediated degradation (in case of p27/KIP1). Further studies are needed to access these possibilities. It is also possible that the apoptosis induction by sanguinarine is a process independent from $G_1$-phase arrest. Further studies are needed to clarify this assumption.

One major finding of this study is that sanguinarine has been shown to cause cell cycle blockade and apoptosis of human prostate cancer cells irrespective of their androgen status. This is an important finding because prostate cancer is known to undergo a transition from an early ''androgen-sensitive'' form of cancer to a late (metastatic) ''androgen-insensitive'' cancer, and at the time of clinical diagnosis, most prostate cancer represent a mixture of androgen-sensitive and androgen-insensitive cells. Therefore, the key to the control of prostate cancer seems to lie in the elimination of both types of prostate cancer cells (without affecting the normal cells) via mechanism-based preventive/therapeutic approaches. To our knowledge, this is the first study showing the involvement of cki-cyclin-cdk machinery during cell cycle arrest and apoptosis of prostate cancer cells by sanguinarine. These results suggest that sanguinarine may be developed as an agent for the management of prostate cancer.

#### References

**1.** Jemal A, Tiwari RC, Murray T, et al. Cancer statistics, 2004. CA Cancer J Clin 2004;54:8–29.

**2.** Tang DG, Porter AT. Target to apoptosis: a hopeful weapon for prostate cancer. Prostate 1997;32:284–93.

**3.** Denmeade SR, Lin XS, Isaacs JT. Role of programmed (apoptotic) cell death during the progression and therapy for prostate cancer. Prostate 1996;28:251–65.

**4.** Mukhtar H, Ahmad N. Cancer chemoprevention: future holds in multiple agents. Toxicol Appl Pharmacol 1999;158:207 – 10.

**5.** Hail N Jr, Lotan R. Examining the role of mitochondrial respiration in vanilloid-induced apoptosis. J Natl Cancer Inst 2003;94:1281 – 92.

**6.** Chaturvedi MM, Kumar A, Darnay BG, Chainy GB, Agarwal S, Aggarwal BB. Sanguinarine (pseudochelerythrine) is a potent inhibitor of NF-κB activation, IκBα phosphorylation, and degradation. J Biol Chem 1997;27:30129 – 34.

**7.** Walterova D, Ulrichova J, Valka I, Vicar J, Vavreckova C, Taborska E, et al. Benzo[*c*]phenanthridine alkaloids sanguinarine and chelerythrine: biological activities and dental care applications. Acta Univ Palacki Olomuc Fac Med 1995;139:7 – 16.

**8.** Mandel ID. Chemotherapeutic agents for controlling plaque and gingivitis. J Clin Periodontol 1998;15:488 – 98.

**9.** Mandel ID. Antimicrobial mouthrinses: overview and update. J Am Dent Assoc 1994;125 Suppl 2:2S – 10S.

**10.** Ahmad N, Gupta S, Husain MM, Heiskanen KM, Mukhtar H. Differential antiproliferative and apoptotic response of sanguinarine for cancer cells versus normal cells. Clin Cancer Res 2000;6:1524 – 8.

**11.** Adhami VM, Aziz MH, Mukhtar H, Ahmad N. Activation of prodeath Bcl-2 family proteins and mitochondrial apoptosis pathway by sanguinarine in immortalized human HaCaT keratinocytes. Clin Cancer Res 2003;9:3176 – 82.

**12.** Debiton E, Madelmont JC, Legault J, Barthomeuf C. Sanguinarine-induced apoptosis is associated with early and severe cellular glutathione depletion. Cancer Chemother Pharmacol 2003;51:474 – 82.

**13.** Ding Z, Tang SC, Weerasinghe P, Yang X, Pater A, Liepins A. The alkaloid sanguinarine is effective against multidrug resistance in human cervical cells via bimodal cell death. Biochem Pharmacol 2002;63: 1415 – 21.

**14.** Weerasinghe P, Hallock S, Tang SC, Liepins A. Role of Bcl-2 family proteins and caspase-3 in sanguinarine-induced bimodal cell death. Cell Biol Toxicol 2001;17:371 – 81.

**15.** Weerasinghe P, Hallock S, Tang SC, Liepins A. Sanguinarine induces bimodal cell death in K562 but not in high Bcl-2-expressing JM1 cells. Pathol Res Pract 2001;197:717 – 26.

**16.** Weerasinghe P, Hallock S, Liepins A. Bax, Bcl-2, and NF-κB expression in sanguinarine induced bimodal cell death. Exp Mol Pathol 2001;71:89 – 98.

**17.** Buolamwini JK. Cell cycle molecular targets in novel anticancer drug discovery. Curr Pharm Des 2000;6:379 – 92.

**18.** Collins K, Jacks T, Pavletich NP. The cell cycle and cancer. Proc Natl Acad Sci USA 1997;94:2776 – 8.

**19.** Hajduch M, Havlieek L, Vesely J, Novotny R, Mihal V, Strnad M. Synthetic cyclin dependent kinase inhibitors. New generation of potent anti-cancer drugs. Adv Exp Med Biol 1999;457:341 – 53.

**20.** McDonald ER, El-Deiry WS. Cell cycle control as a basis for cancer drug development. Int J Oncol 2000;16:871 – 86.

**21.** Sherr CJ. Cancer cell cycles. Science 1999;274:1672 – 7.

**22.** Sherr CJ, Roberts JM. CDK inhibitors: positive and negative regulators of $G_1$-phase progression. Genes Dev 1999;13:1501 – 12.

**23.** Ahmad N, Feyes DK, Nieminen AL, Agarwal R, Mukhtar H. Green tea constituent epigallocatechin-3-gallate and induction of apoptosis and cell cycle arrest in human carcinoma cells. J Natl Cancer Inst 1997;89: 1881 – 6.

**24.** Jacks T, Weinberg RA. Cell-cycle control and its watchman. Nature 1999;381:643 – 4.

**25.** Macleod KF, Sherry N, Hannon G, Beach D, Tokino T, Kinzler K, et al. p53-dependent and independent expression of p21 during cell growth, differentiation, and DNA damage. Genes Dev 1995;9:935 – 44.

**26.** Sherr CJ. $G_1$ phase progression: cycling on cue. Cell 1994;79:551 – 5.

**27.** Sherr CJ, Roberts JM. Inhibitors of mammalian $G_1$ cyclin-dependent kinases. Genes Dev 1995;9:1149 – 63.

**28.** Kasibhatla S, Tseng B. Why target apoptosis in cancer treatment. Mol Cancer Ther 2003;2:573 – 80.

**29.** Gleave M, Bruchovsky N, Goldenberg SL, Rennie P. Intermittent androgen suppression for prostate cancer: rationale and clinical experience. Eur Urol 1998;34:37 – 41.

**30.** Weisburger JH. World wide prevention of cancer and other chronic diseases based on knowledge of mechanisms. Mutat Res 1998;402: 331 – 7.

**31.** Fisher DE. Pathways of apoptosis and the modulation of cell death in cancer. Hematol Oncol Clin North Am 2001;15:931 – 56.

**32.** Kanduc D, Mittelman A, Serpico R, Sinigaglia E, Sinha AA, Natale C, et al. Cell death: apoptosis versus necrosis. Int J Oncol 2002;21:165 – 70.

**33.** Ferreira CG, Epping M, Kruyt FA, Giaccone G. Apoptosis: target of cancer therapy. Clin Cancer Res 2002;8:2024 – 34.

**34.** Penn LZ. Apoptosis modulators as cancer therapeutics. Curr Opin Invest Drugs 2001;2:684 – 92.

**35.** Vermeulen K, Berneman ZN, Van Bockstaele DR. Cell cycle and apoptosis. Cell Prolif 2003;36:165 – 75.

**36.** King KL, Cidlowski JA. Cell cycle regulation and apoptosis. Annu Rev Physiol 1998;60:601 – 17.

**37.** Morgan SE, Kastan MB. p53 and ATM: cell cycle, cell death, and cancer. Adv Cancer Res 1997;71:1 – 25.

**38.** Sandhu C, Slingerland J. Deregulation of the cell cycle in cancer. Cancer Detect Prev 2000;24:107 – 18.

**39.** Hartwell LH, Kastan MB. Cell cycle control and cancer. Science 1994;266:1821 – 8.

**40.** Kastan MB, Canman CE, Leonard CJ. P53, cell cycle control and apoptosis: implications for cancer. Cancer Metastasis Rev 1995;14:3 – 15.

**41.** Molinari M. Cell cycle checkpoints and their inactivation in human cancer. Cell Prolif 2000;33:261 – 74.

**42.** McDonald ER, El-Deiry WS. Cell cycle control as a basis for cancer drug development. Int J Oncol 2000;16:871 – 86.

**43.** Owa T, Yoshino H, Yoshimatsu K, Nagasu T. Cell cycle regulation in the $G_1$ phase: a promising target for the development of new chemotherapeutic anticancer agents. Curr Med Chem 2000;8:1487 – 503.

**44.** Jones SM, Kazlauskas A. Connecting signaling and cell cycle progression in growth factor-stimulated cells. Oncogene 2000;19:5558 – 67.

**45.** Zwicker J, Muller R. Cell cycle-regulated transcription in mammalian cells. Prog Cell Cycle Res 1995;1:91 – 9.

**46.** Pavletich NP. Mechanisms of cyclin-dependent kinase regulation: structures of cdks, their cyclin activators, and CIP and INK4 inhibitors. J Mol Biol 1999;287:821 – 8.

**47.** Ortega S, Malumbres M, Barbacid M. Cyclin D-dependent kinases, INK4 inhibitors and cancer. Biochim Biophys Acta 2002;1602:73 – 87.

**48.** Gartel AL, Serfas MS, Tyner AL. p21-negative regulator of the cell cycle. Proc Soc Exp Biol Med 1996;213:138 – 49.

**49.** Dotto GP. p21(WAF1/Cip1): more than a break to the cell cycle? Biochim Biophys Acta 2000;1471:43 – 56.

**50.** Macri E, Loda M. Role of p27 in prostate carcinogenesis. Cancer Metastasis Rev 1998;17:337 – 44.

**51.** Lukas J, Bartkova J, Rohde M, Strauss M, Bartek J. Cyclin D1 is dispensable for $G_1$ control in retinoblastoma gene-deficient cells independently of cdk 4 activity. Mol Cell Biol 1995;15:2600 – 11.

**52.** Bartkova J, Lukas J, Bartek J. Aberrations of the $G_1$- and $G_1$-S-regulating genes in human cancer. Prog Cell Cycle Res 1997;3:211 – 20.

**53.** Lloyd RV, Erickson LA, Jin L, Kulig E, Qian X, Cheville JC, et al. p27kip1: a multifunctional cyclin-dependent kinase inhibitor with prognostic significance in human cancers. Am J Pathol 1999;154:313 – 23.

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**Preclinical evaluation of plant alkaloid sanguinarine against prostate cancer development in a nude mice xenograft model.**

**Short Title:**
Sanguinarine and Prostate Cancer

*Moammir H. Aziz, Imtiaz A. Siddiqui, Haseeb Ahsan, Shannon R. Reagan-Shaw, Nihal Ahmad.* University of Wisconsin, Madison, WI

Prostate Cancer (PCa) is one of the most common malignancies of men in the USA and many other countries in world. Each year ~543,000 new cases are reported worldwide and the disease kills 200,000 (mostly older men) in developed countries. The traditional surgery and therapy has not been successful in the management of PCa. Therefore, the search for novel agents and approaches for the treatment of PCa continues. Chemopreventive strategies, especially with naturally occurring plant-based agents, have shown promise for prevention as well as treatment of PCa. We recently demonstrated that sanguinarine(13-methyl[1,3]benzodioxolo[5,6-c]-1,3-dioxolo [4,5-i]phenanthridinium), derived from the root of *Sanguinaria Canadensis* and other poppy-fumaria species, causes cell cycle arrest and apoptotic death of human prostate carcinoma cells (*Mol Cancer Ther 3: 933-940, 2004*). Sanguinarine is a benzophenanthridine alkaloid and a structural homologue of chelerythrine and has been shown to possess anti-microbial, antioxidant and anti-inflammatory properties. In this study, we determined the chemopreventive and therapeutic potential of sanguinarine against prostate cancer *in vivo* in athymic nude mice implanted with androgen responsive human prostate carcinoma CW22Rv1 cells. For this purpose, CW22Rv1 cells ($1x10^6$ cells in 50 µl RPMI + 50 µl Matrigel) were implanted in athymic nude mice by a sub-cutaneous injection on left and right sides, below the shoulders (2 tumors/mouse). The animals were treated with sanguinarine (1 or 5 mg/kg body weight in 0.2 ml PBS, five days a week) by intra-peritoneal injection either one week post cell implantation to establish the preventive potential or after the development of a sizable tumor (200 $mm^3$) to examine the therapeutic potential. The control animals received vehicle only. Our data demonstrated that sanguinarine (both pre- and post- treatments) resulted in a highly significant inhibition in the rate of tumor growth as assessed by a regression analysis. Further, the Kaplan-Meier Analysis demonstrated that in sanguinarine treated animals (post-treatment), the rate of tumor growth (to reach to a 1000 $mm^3$ target volume) was significantly delayed. Furthermore, treatment of mice with sanguinarine (both pre- and post-tumor) resulted in a significant reduction in serum levels of prostate-specific antigen (PSA) in nude mice implanted with CWR22Rv1 cells. This study, for the first time, demonstrated the chemopreventive and therapeutic effects of sanguinarine against PCa development under *in vivo* situations. Based on our data, we suggest that sanguinarine is a promising candidate for chemoprevention and/or intervention against PCa.
**Author Disclosure Block: M.H. Aziz**, None; **I.A. Siddiqui**, None; **H. Ahsan**, None; **S.R. Reagan-Shaw**, None; **N. Ahmad**, None.

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